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# Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment*

# Oestrogenic potency of nonylphenol *in vivo*—a case study to evaluate the relevance of human non-occupational exposure

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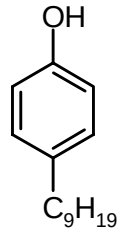
## INTRODUCTION

4-Nonylphenol (NP) is an alkylphenolic compound that is mainly used as an intermediate in the chemical manufacturing industry (Structure and physico-chemical data are given in Figure 1). NP is used directly for example to label tax-favoured light fuel oil, as an preservative agent, in the tanning industry, etc.. In the form of NP-phosphites, it finds use as an co-stabilizer and antioxidant in various polymers like rubber and plastics; in the form of NP metal salts it is used as a stabilizer in PVC (ref. 1). But the predominant use of NP is in the form of NP polyethoxylates (NPnEO) with variable ethoxylate chain length as emulsifying, dispersing, wetting and foaming agent, e.g. in paints, in cosmetics, in agrochemicals, in textile manufacturing, in metal finishing, as spermicide, and as additive in lubricating oils (refs 1, 2). In 1994, the annual use of NP was estimated to be 78 497 tons/year solely in Europe (ref. 3).




**4-Nonylphenol**
CAS: 84852-15-3
25154-52-3

| | |
|---|---|
| Molecular weight: | 220.34 g/mol |
| p$K_a$ (estimated): | 10.28 |
| Vapour pressure: | 0.3 Pa (25 °C) |
| Water solubility: | 6 mg/L |
| Partition coefficient: | 4.48 |

**Fig. 1.** Structure and physico-chemical properties of 4-nonylphenol.

The oestrogenic properties of p-alkylated phenolic compounds were recognized as early as 1938 (refs 4, 5). NP binds to the oestrogen receptor and exerts oestrogenic actions *in vivo* and *in vitro* (refs 6–9).

Human exposure routes are diverse. Exposure via contaminated foods and drinking water, but also via dermal absorption after the application of NP-containing products, or via inhalation could occur (refs 10–12). The question arises whether the human exposure to NP and its derivatives could result in blood and organ concentrations great enough to elicit oestrogenic effects.

In this study, we assess the oestrogenic potency of NP and perform a risk calculation for non-occupationally exposed humans. This calculation is based on the estimated daily intake of NP, the relative oestrogenic potency as evaluated from different *in vivo* and *in vitro* systems, the pharmacokinetic

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behavior in humans, and comparison of the oestrogenic burden in blood and organs with the phyto-oestrogen genistein and 17β-oestradiol, respectively.

## OESTROGENIC ACTION AND POTENCY OF NONYLPHENOL

The oestrogenic activity of NP has been investigated in a number of *in vitro* and *in vivo* studies. NP is capable to displace competitively tritiated 17β-oestradiol from the human and fish oestrogen receptor (Table 1), indicating an oestrogenic action directly via the oestrogen receptor with a potency of 1000 to 3000 times less than 17β-oestradiol itself (refs 13–16).

**Table 1.** Oestrogenic potency of NP (competitive displacement of 17β-oestradiol from the oestrogen receptor)

| Oestrogen receptor (species) | Relative potency | Reference |
|---|---|---|
| Rainbow trout | 0.0007 | (13) |
| Rainbow trout, roach | 0.0003 | (14) |
| Rainbow trout | 0.0009 | (9) |
| MCF-7 cell line | 0.0003 | (15) |

*In vitro*, a weak oestrogenic activity of NP was shown in an recombinant yeast system containing plasmids carrying oestrogen-responsive sequences that controlled the expression of the reporter gene lac-Z (encoding the enzyme β-galactosidase) (ref. 16). Similar results were observed in primary trout hepatocytes, using the expression of vitellogenin / vitellogenin mRNA as biomarkers (refs 13, 17), and in transfected avian cells using the reporter gene EREBLCAT (ref. 9). Positive results were also seen in human MCF-7 breast cancer cell lines with up to 100% serum, and in ZR-75 breast cancer cell lines regarding cell growth as endpoint (refs 7, 9, 18–20). From this studies it can be deduced that NP is about 1000–10 000 times less potent than 17β-oestradiol and the lowest effective extracellular concentrations is supposed to be about 100 nM–1 µM (Table 2).

**Table 2.** Potency of NP relative to oestradiol *in vitro*

| *In vitro* system | Relative potency | Minimum effective concentration | Reference |
|---|---|---|---|
| Recombinant yeast | 0.0002 | 1 µM | (44) |
| Recombinant yeast | 0.0001 | 400 nM | (16) |
| Primary trout hepatocytes | - | 100 nM | (13) |
| Primary trout hepatocytes | 0.000009 | 1–10 µM | (17) |
| MCF-7 cell line | 0.0001 | 1 µM | (18) |
| MCF-7 cell line | 0.00039 | – | (20) |
| Serum modified | 0.00026 | | |
| MCF-7 cell line | 0.000003 | 10 µM | (7) |
| MCF-7 cell line* | 0.001 | 10 nM | (19) |
| MCF-7 cell line* | 0.0001 | 100 nM | (19) |
| MCF-7; ZR-75 cell line | 0.0001 | 10 µM | (9) |
| Transfected avian cells | <0.0001 | 1–10 µM | (9) |

*Different cell stocks

In *in vivo* experiments in rodents NP was 10 000–1000 000 times less potent than oestradiol or diethylstilbestrol (Table 3). In the uterine assay in ovariectomized rats, the endometrial mitotic index after application of 20 mg NP was lower than that observed with 1.25 µg 17β-oestradiol (ref. 21). Induction of uterine growth was found at a dose of = 1 mg (ref. 22). Stimulation of increased uterine vascular permeability in ovariectomized mice was observed already at 4 hours after application (ref. 23).

NP also affected the proliferation, cell-cycle kinetics, and differentiation of the mammary gland (ref. 24). Since in all *in vivo* investigations NP was applied subcutaneously, the possibility of reduced bioavailability following oral application was not addressed. Interestingly, female rats treated in combination with oestradiol and NP had lower uterine weights than those treated with oestradiol alone, indicating an action as partial antagonist (ref. 24).

**Table 3.** *In vivo* potency of NP relative to 17β-oestradiol in female rats

| Oestrogenic end point | Relative potency | Reference |
|---|---|---|
| Proliferative effect in mammary glands | 0.00001–0.000001* | (24) |
| Uterine weight | 0.0001–0.00005 | (22) |
| Uterine vascular permeability | 0.00001 | (23) |
| Endometrial mitotic index | <0.00006 | (21) |

*Potency relative to DES

A 90 day subchronic toxicity study was conducted in rats. NP was administered at dietary concentrations of 0, 200, 650, or 2000 p.p.m. which corresponded to approximate dietary intakes of 0, 15, 50, or 150 mg/kg bw/day, respectively (ref. 25). The study included endocrine-sensitive measurements to gain information on potential hormonal effects; i.e. oestrus cyclicity, sperm counts, motility and morphology. Effects were limited to slight decreases in body weight gain and food consumption, increased reversible kidney weights and decrease in renal hyaline globules in males with a lack of correlating clinical or histopathological changes at 2000 p.p.m.. The kidney effects were regarded as with questionable toxicological significance to humans. No changes were observed for oestrus cycling, sperm evaluations, or effects on endocrine organs up to the highest dose at 2000 p.p.m.. The NOAEL was concluded to correspond to 50 mg/kg bw/day.

NP was tested for reproductive effects over three generations in rats at a concentration of 0, 200, 650, and 2000 p.p.m. in the diet (ref. 26). The study found effects including male and female reproductive changes in rats dosed at 650 p.p.m. and above, based on decreased epididymal sperm density and testicular spermatid head counts in males (F2 generation only), and increased oestrus cycle length and decreased ovarian weights observed in females. These changes were not linked to alterations in fertility. The reproductive changes were seen only concomitant with body weight changes and renal lesions. No adverse reproductive effects were observed at 200 p.p.m., which means a NOEL for reproductive toxicity of 15 mg/kg bw/day. NP was concluded to be a reproductive toxicant at concentrations of equal or greater than 650 p.p.m., that also exhibited signs of systemic toxicity.

## DISTRIBUTION AND PHARMACOKINETIC IN PRIMARY MOUSE HEPATOCYTE CULTURES

We examined the distribution and the pharmacokinetic behaviour of NP in primary mouse hepatocyte cultures in order to interpret quantitatively published *in vitro* studies (ref. 27). Since the oestrogenic response is usually reported on the basis of extracellular medium concentration, the actual effective cell concentrations are unknown. In our studies, $10^{-5}$ M NP was added to the medium of primary mouse hepatocytes and the time course of the concentration of NP in the medium and in the cell fraction measured up to 48 hours. In the medium, a rapid decrease of NP was observed. In the cells, a slow decline rather than an accumulation was found (Table 4). Since only about 5% of the applied amount was present after 48 h in the cell medium and cell fraction as parent NP, a rapid metabolism was deduced, which is consistent with the studies from Certa where a rapid metabolism of the structurally related compound 4-octylphenol in rat hepatocyte fractions was found (ref. 28). A linear extrapolation to $10^{-7}$ M NP, which was reported as the minimum concentration with an oestrogenic effect *in vitro*, would thus correspond to a cell concentration of about 100 ng/g cells.

**Table 4.** Concentration of NP in the cell fraction of mouse cell cultures after addition of $10^{-5}$ M NP (ref. 27).

| Time [hours] | Average cell concentration µg NP/mg cells | Number of plates |
|---|---|---|
| 2 | 41.9 | $n = 2$ |
| 8 | 18.2 | $n = 2$ |
| 24 | 11.1 | $n = 4$ |
| 48 | 9.2 | $n = 5$ |

## PHARMACOKINETIC BEHAVIOR IN HUMANS

The pharmacokinetic behavior of NP was investigated in two human volunteers (ref. 29). Stable isotope labelled $^{13}C_6$-NP was used in order to avoid background contamination problems and to keep the dose low.

$^{13}C_6$-NP was applied orally to volunteer A at a dose of 5 mg (67 µg/kg body weight) and intravenously to volunteer B at a dose of 1 mg (14 µg/kg body weight). An elimination half-life of $^{13}C_6$-NP from blood both after oral and intravenous application of 2–3 hours was found. The substance seems to distribute into the lipid phase of the body within 2–3 hours. The bioavailability (the amount of parent NP entering systemic circulation) after oral administration was found to be only 20%, calculated by comparison of the area under the curve (AUC) after intravenous and after oral application. This is a consequence of a rapid hepatic metabolism since less than 1% of the applied dose was recovered in the faeces as parent NP.

## ESTIMATION OF HUMAN EXPOSURE

The maximum oral daily intake of NP by non-occupationally exposed people is estimated to be less than 0.16 mg/day (Table 5). This estimation was based on literature data of NP concentrations in food and drinking water.

**Table 5.** Estimation of the daily intake of NP through food and drinking water based on literature data.

| Food | Concentration on the basis of wet weight | Estimated daily intake | Reference |
|---|---|---|---|
| Migration from PVC / plastics | ≤0.1 mg/kg | 1 kg food | (12) |
| Duck / fish | 0.02–0.3 mg/kg | 100 g | (10) |
| Drinking water | ≤0.002 mg/L | 3000 g | (11, 45) |
| Estimated daily intake: | | ≤ 0.16 mg | |

## ASSESSMENT OF NON-OCCUPATIONAL EXPOSURE

### Comparison of the daily intake with in vivo experiments

Comparison of the estimated daily intake of <0.16 mg/day on non-occupationally exposed humans with the NOAEL of 50 mg/kg bw derived from the subchronic toxicity study in rats (ref. 25) results in a safety factor of about 20 000. Since no treatment-related effects were observed in this study for all endocrine sensitive measurements up to the highest dose, the safety factor for oestrogenic effects is considered to even be higher.

Comparison of the estimated daily intake of non-occupationally exposed humans with the NOEL of 15 mg/kg bw/day for reproductive toxicity derived from the multigeneration reproduction study in rats (ref. 26) results in a safety factor of about 5000.

### Comparison of blood concentrations with 17β-oestradiol levels

Using the pharmacokinetic data from human volunteers, blood concentrations after oral ingestion can be calculated. A maximum peak blood concentrations of 10 pg/mL NP would result after an intake of 0.15 mg NP distributed among three meals per day. These peak concentrations would decrease with a half-time of 2–3 hours. Comparison of the calculated peak concentrations in blood with those of 17β-oestradiol (30 pg/mL in human males) and taking into account a difference in oestrogenic potency of 10 000 results in an oestrogenic burden from NP of at least a factor of 30 000 below that of endogenous oestrogens.

### Comparison of organ concentrations with minimum oestrogenic concentrations

Assuming a distribution of the systemically available NP into the lipid phase of the body (20 kg fat) an organ concentration of 25 pg/g organ can be calculated (intake 0.05 mg; bioavailability: 20%; lipid content of organs: 5%). A comparison with the minimum concentration necessary to induce an oestrogenic effect (100 ng/g cells as derived from our *in vitro* investigations) again suggests a factor of 4000 below the no effect level.

### Comparison with the phyto-oestrogen genistein

Genistein is an isoflavonoid substance of plant origin closely related in structure to 17β-oestradiol. Genistein occurs in different plants, particularly soy beans (up to 3g/kg dry weight), as well as in alfalfa, pinks, *Prunus* species, red clover and black beans (ref. 30).

The binding affinity of genistein to the oestrogen receptor was found to be 50–10 000 times less than that of oestradiol (refs 31, 32). *In vitro*, the oestrogenic potency in the MCF-7 breast cancer cell line and in fish hepatocytes was 1000–10 000 times less than 17β-oestradiol (refs 33, 34). Genistein stimulated *in vivo* uterine growth in ovariectomized mice, sheep and rats (refs 23, 31). The potency of genistein in reducing the anogenital distance in the offspring of rats treated subcutaneously was about a factor of 1000 less than 17β-oestradiol (ref. 35). In premenopausal women, the daily consumption of 60 g textured soy proteins for one month, but not consumption of soy protein from which the isoflavones had been removed, significantly decreased follicle stimulating hormone and luteinizing hormone levels, and increased menstrual cycle length (ref. 36). As well as exhibiting oestrogen-like effects, genistein may also play a role in retarding cancer growth, due to other than oestrogenic effects (ref. 32).

In Western Europe and North America the isoflavonoid intake is estimated to be a few milligrams (ref. 37) and in the Asian countries 50–100 mg/d (refs 30, 38), which is generally regarded as safe. Assuming an isoflavonoid intake of 10 mg in Western Europe and North America and 100 mg/d in Asia and that genistein accounts for 70% of total isoflavonoid content (ref. 30), we may estimate an average daily genistein intake of 7 and 77 mg/d, respectively. The bioavailability of genistein is estimated to be 13–35 % (ref. 39). The blood elimination half-life in humans was found to be 7–8 hours (ref. 40). Blood levels of 0.1–0.6 ng genistein/mL plasma (free and sulfate fraction) were detected in humans in Europe and up to 2.1 ng/mL in Japan (refs 36, 41).

The daily intake of NP and genistein, which exhibit both weakly oestrogenic and partially anti-oestrogenic properties, their oestrogenic potency, blood levels and oestrogenic burden in comparison to oestradiol are evaluated (Table 6) The possible influence of protein binding to the oestrogenic burden is not taken into account, since *in vivo* experiments assessing the oestrogenic potency of NP relative to 17β-oestradiol do not give any indication of a significantly increased oestrogenic activity compared to *in vitro* results (refs 42, 43). The daily intake of genistein exceeds the intake of NP by several orders of magnitude and its oestrogenic potency based on extracellular concentrations is higher than that of NP.

The oestrogenic burden from genistein in humans is calculated to be 100 to 2000 fold higher than that from NP.

**Table 6.** Comparison of daily intake, the oestrogenic potency and resulting oestrogenic burden in blood of NP, genistein and the endogenous oestrogen 17β-oestradiol

| | Daily intake [mg/d] | Oestrogenic potency relative to oestradiol | Blood levels [pg/mL] | Oestrogenic burden in blood |
|---|---|---|---|---|
| nonylphenol | ≤0.16 | ≈10–4 | <10 | 0.00003 |
| genistein | 7–77 | ≈10–3 | 100–2100 | 0.003–0.07 |
| 17β-oestradiol | – | 1 | 30* | 1 |

* Levels in human adult males (ref. 46)]

## CONCLUSIONS

The estimated daily intake of non-occupationally exposed humans does not represent a health risk. This was shown by the following comparisons:

**1** Comparison of the estimated daily intake with the NOAEL of a 90-day oral toxicity study including oestrogenic sensitive end points, and with the NOEL of a multigeneration reproduction study, results in a safety factor of at least 5000.

**2** Comparison of calculated NP organ concentrations with estimated cellular oestrogenic no-effect concentrations results in a safety factor of 4000.

**3** Comparison of the oestrogenic burden of NP in blood with that of 17β-oestradiol results in a difference of about 30 000. Furthermore, the oestrogenic burden of the phyto-oestrogen genistein in humans, which is generally regarded as safe, is at least a factor of 100 higher than that of NP.

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